Relationship between Sap prevalence and biofilm formation among resistant clinical isolates of *Candida albicans*

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Abstract

Background: Fungal infections represent a serious health problem especially in immunocompromised individuals. *Candida albicans* is the most common fungi that cause superficial and systemic infections with high mortality rates. Anti-fungal resistance of *C. albicans* may be attributed to its virulence. Biofilm formation and proteolytic activity are major virulence determents that may influence both pathogenicity and anti-fungal resistance of *Candida albicans*.

Objective: This work studied the relation between biofilm formation, proteolytic activity and prevalence of some Sap genes with reduced susceptibility of *C. albicans* to different anti-fungal agents.

Methods: Fifty three *C. albicans* strains isolated from patients with systemic infections, identified by germ tube, chromogenic agar and confirmed by PCR, were subjected to evaluate their proteolytic activity, the degree of biofilm production and the prevalence of Sap9 and Sap10 genes. The susceptibility of the isolates was determined by disk diffusion method against five antifungal drugs.

Results and conclusion: Four of the *C. albicans* isolates were resistant to 3 anti-fungal drugs, strong biofilm producer, have proteolytic activity and contain either Sap9 or Sap10 or both. Conclusively, although anti-fungal resistance among the isolates was rare, a relation between the anti-fungal resistance and some major virulence factors was evidently proved in this study.

Keywords: *Candida albicans*, resistance, biofilm, proteolytic, Sap.

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Introduction

Candida infections have a significant rise in morbidity and mortality, especially in recent years due to the continuous increase in the number of immunosuppressive patients[1]. *Candida albicans* is determined as the major human pathogen in the genus *Candida*[2]. *C. albicans* is classified as commensal fungi that present in` many anatomical sites of the human body[3]. *C. albicans* can cause oral and vaginal infections as well as systemic diseases[4]. The rise in the incidence of Candida infections is complicated by the antimicrobial resistance and the limited number of available anti-fungal drugs[5].

The ability of Candida to cause infection depends mainly on its intrinsic virulence attributes[6]. *C. albicans* cause critical problems because it has more virulence factors than non-*Candida albicans* isolates[7]. *C. albicans* has several virulence factors including phenotyping changes, biofilm formation, and production of harmful substances to cells, such as haemolysins, phospholipases and proteases as well as the ability to resist hydrogen peroxide[8].

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Among the hydrolytic enzymes that secreted by *Candida spp*. are the aspartic proteases which represent one of the major virulence determinants as they have a potential role in pathogenicity through facilitating the invasion and counteracting the host defense system[9].

Secreted aspartic proteases (Saps), encoded by a family of 10 sap genes (sap1–sap10) which have a vital role in virulence of *C. albicans* by degrading host tissue proteins as well as adhere to epithelial host tissue[10]. Saps have a broad substrate specificity of human proteins such as albumin, haemoglobin, keratin, collagen, laminin, fibronectin, mucin and almost all immunoglobulins, including immunoglobulin A, which is resistant to the majority of bacterial proteases[11]. A correlation between the expansion of sap genes and the transition from commensal to pathogenic microorganisms has been reported[12]. Non-pathogenic *Candida spp*. usually has fewer genes encoding Sap than pathogenic species and this fact was confirmed by gene sequencing. However, this rule cannot be applied to species such as *C. glabrata* or *C. krusei*, which do not possess any sap genes[12]. On the other hand, there is a correlation reported between Sap proteinase production and biofilm formation[13].

Biofilms are defined as self-derived extracellular matrix that produced by the microbial population attached to abiotic surface including biomedical devices or biotic surfaces including oral and vaginal mucosa[14,15]. *C. albicans* present in biofilm structure show a decrease in susceptibility to some anti-fungals as well as a reduction in killing by the host immune system[16]. Sap 9 and Sap10 enzymes maintain cell surface integrity of the *Candida* cell wall, and promote biofilm formation[17].

This study aims to correlate between sap9 and sap10 prevalence and its role in biofilm formation and drug resistance among clinical isolates of *C. albicans*.

Materials and methods

Candida albicans isolates

All the clinical specimens were collected under ethical standards from different Departments at Mansoura University Hospitals, and identified according to Cheesbrough[18]. Briefly, the specimens were inoculated on Sabouraud dextrose agar (SDA) plates and incubated at 37 ˚C for 24-48 hr. The suspected colonies of *C. albicans* were examined for their colonial morphology, Gram staining, germ tube formation, culture characteristics on *Candida* chromogenic agar (Pronadisa Co., Madrid, Spain) and the identification was confirmed by PCR[19], The standard strain of *Candida albicans* (ATCC 10231) was included in this study.

Determination of anti-fungal susceptibility by disk diffusion method

Candida albicans isolates were tested for their susceptibility to different anti-fungal agents by disk diffusion method according to CLSI guideline[20]. The tested disks that include; Amphotericin B (AMB, 10μg), Fluconazole (FLU, 25μg), Voriconazole (VOR, 1μg), Fluorocytosine (5FC, 1μg), Caspofungin (CASP, 5μg); were obtained from Bioanalyse, Turkey.

Phenotypic detection of aspartyl proteinase activity in *C. albicans* isolates

Briefly, *Candida* isolates were cultured in YEPD medium (2% glucose, 1% yeast extract, and 2% Bactopeptone) and then they induced to secrete proteinases onto the bovine serum albumin (BSA) agar. Filter paper disks, 6 mm diameter, were dipped into a suspension of *Candida* culture at a density of 10^7 cell mL^{-1} (0.5 McFarland) in YEPD medium and applied to BSA agar plate. A maximum of 4 disks was used for each 9 cm diameter plate. The plates were incubated at 28°C for 7 days. The plates were observed daily for opacity around the disks, the opacity caused by albumin precipitation was observed for subsequent clearing due to hydrolysis by the acid proteinases of the fungi. The millimetric zones were evaluated as negative (–) for no clearance, positive (+) for mild activity (lysis zone of 1-2 mm around the disk), and double-positive (++) for strong activity (lysis zone of 3-5 mm). The standard strain was used as positive controls, and the experiment was performed in triplicate[21].

Genotypic detection of Sap9 and Sap10 in Candida albicans isolates using PCR

DNA of Candida albicans was extracted by colony PCR method[22]. The primers used for detection of sap9 and sap10 in *C. albicans* (SAP9F: 5` ATTTACTCCACAGTTTATATCACTGAAGGT3`, SAP9R: 5` CCACCAGAACCACCCTCAGTT 3`, SAP10F: 5` CCCGGTATCCAATAGAATCGAA3` and SAP10R: 5` TCAGTGAATGTGACGAATTTGAAGA 3`) were

purchased from Operon Biotechnologies GmbH Biocompus cologne, Germany[23].

DNA samples were amplified in 25 μL reaction mixture containing 2.5 μL DNA, 12.5 μL my Taq red mix (Bioline Co., UK), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM) and nuclease free water to 25 μL. the cycling conditions include heating at 94°C for 5 min, then 35 cycles of 94°C for 10 sec, 59°C for 20 sec and 72°C for 30 sec and finally heating at 72°C for 3 min. The PCR products as well as GeneRuler 50 bp plus DNA ladder (Thermo scientific, USA) were separated on 1.5% agarose gel, stained with ethidium bromide, visualized by UV transilluminator and photographed.

Detection of biofilm formation in *Candida albicans* isolates

The biofilm formation was performed as described previously[24]. Briefly, a colony from each isolate was obtained from the overnight growth on SDA agar plate and inoculated into 5 mL of Sabouraud broth (SDB). Broths were incubated for 18-20 hr at 37°C. Yeast cells were twice centrifuged (5000 rpm for 5 min) and washed with 0.5 mL phosphate buffer saline (PBS). The cells were re-suspended in 1 mL SD broth and adjusted to concentration of 10^7cells/mL. Then, 200 μL of each isolate suspension was inoculated into individual well of polystyrene 96-well plates, planktonic cells were discarded through three rounds of washing with 200 μL sterile PBS buffer, and the plates dried at room temperature for 45 min. For staining, 150 μL of 0.4% Crystal Violet (CV), was added to each well, after 45 min, supernatant was discarded before adding 150 μL of 95% ethanol to dissolve and/or elute CV from the biofilm for 45 min. Finally, 100 μL of each well was transferred to a new 96-well microtiter plate and the absorbance at 540 nm was determined using a microtiter plate reader Synergy HT (BioTek Instruments, Winooski, VT, USA).

For each strain, the mean OD of four wells was calculated (ODt) and also cut-off OD (ODc) was defined as 3 standard deviations above the mean OD of the negative control. The level of biofilm production was determined as follows: non-biofilm producer (N) ODt ≤ ODc, weak biofilm producer (W) ODc < ODt ≤ 2×ODc, moderate biofilm producer (M) 2×ODc < ODt ≤ 4×ODc and strong biofilm producer (S) ODt > 4×ODc[25].

Results

Identification of *C. albicans* isolates

Fifty three non-duplicate *Candida albicans* clinical isolates were identified in the present study. The isolates were identified as *Candida albicans* by PCR. Meanwhile, 51 isolates gave green colonies of *Candida albicans* on Chromogenic agar and 45 of *Candida albicans* isolates were germ tube positive. On the other hand, 8 isolates of *C. albicans* were germ tube negative (false negative). Among these isolates 30 were from the respiratory tract, 17 from urinary tract and 6 were from blood samples.

Susceptibility to anti-fungals

The data presented in table (1), showed the susceptibility of *Candida albicans* to the different anti-fungal disks. The data revealed that, out of 53 *Candida albicans* isolates five (9.4%) isolates were resistant to three anti-fungal drugs. Approximately, fluconazole and voriconazole show 9.4%, 11.3% resistance while 5 fluorocytosine show 100% resistance. On the other hand, both caspofungin and amphotericin B show no resistance.

Table 1

Antifungals	Number and percentage of *C. albicans* isolates (n=53)					
	R		I		S	
	N	%	N	%	N	%
FLU	5	9.4	2	3.8	46	86.8
VOR	6	11.3	3	5.7	44	83
AMB	0	0	2	3.8	51	96.2
5-FC	53	100	0	0	0	0
CASP	0	0	1	1.9	52	98.1

Proteolytic and biofilm activity

The data presented in Fig. (1A) showed that 8 out of 53 isolates of *C. albicans* (15%) have strong activity of aspartyl proteinase, 27 isolates have mild activity (51%), while 18 isolates (34%) revealed no aspartyl proteinase activity.

The result of biofilm formation shown in Fig. (1B) revealed that 12 (22.6%) isolates out of 53 were strong biofilm forming. Meanwhile, 1 (1.9%) isolate was moderate and 9 (17%) were weak biofilm producers. In addition, 31 (58.5%) isolates were non-biofilm producers.

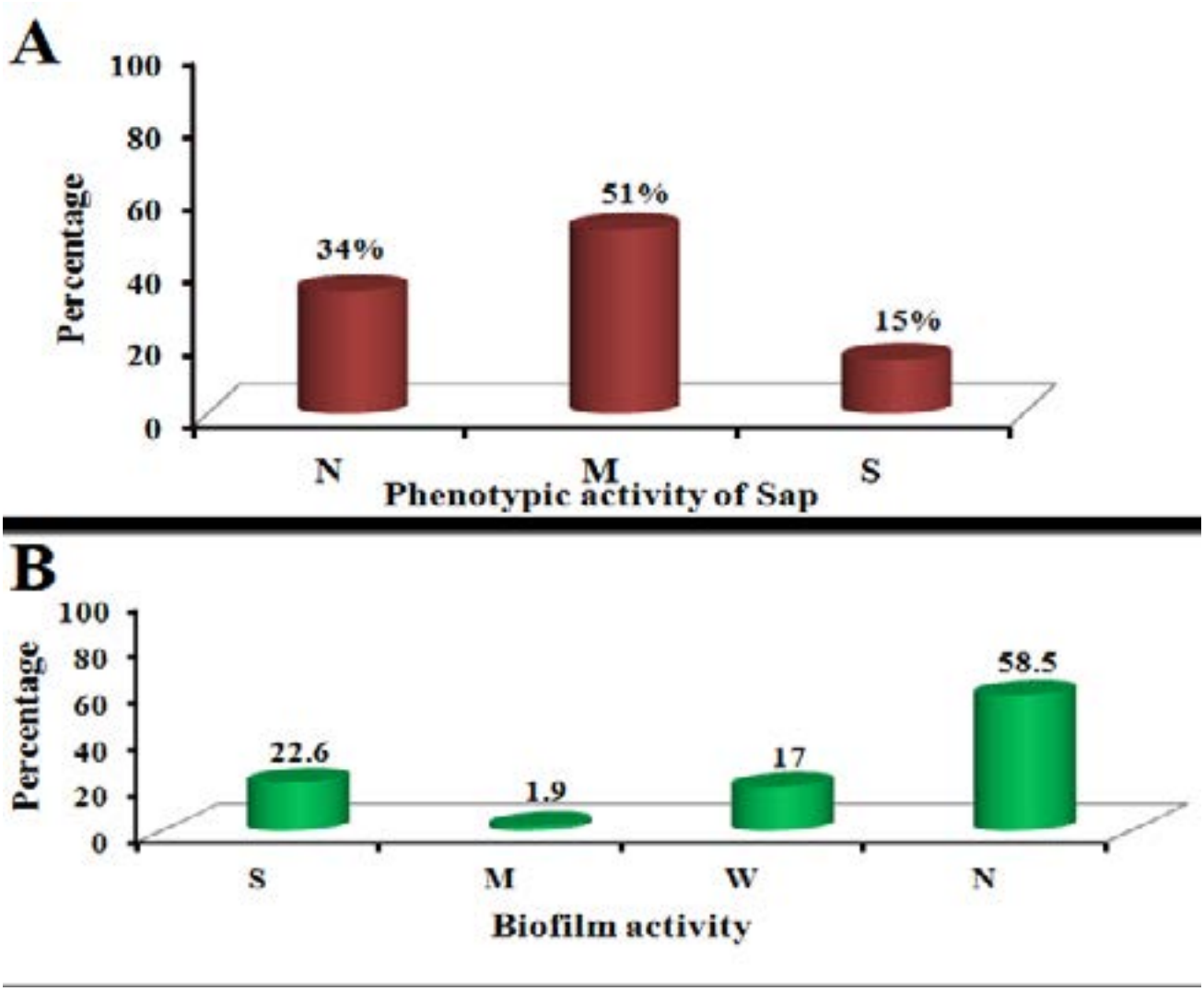

Fig. (1): A: Phenotypic detection of secreted aspartyl protease activity in *C. albicans* isolates by culturing on bovine serum agar (BSA) agar and incubated at 28°C for 7 days and measuring the clearing zone around the growing colony, B: Detection of biofilm activity in *C. albicans* isolates after biofilm growth on microtiter plate containing Sabouraud broth (SDB) for 48 hours and measure the absorbance at 540 nm after staining with crystal violet.

Genotypic detection of Sap in *C. albicans* isolates using PCR

In the present study 53 isolates of *C. albicans* in addition to standard *Candida albicans* strain (ATCC10231) were tested for the presence of Sap9 and 10 genes. Sap9 gene was detected in 45 (84.9%) isolates, while Sap10 gene was detected in 42 (79.2%) isolates. Detection of Sap9 and Sap10 genes in the PCR products of *Candida albicans* isolates on agarose gel at approximately 80 bp, as shown in figure (2).

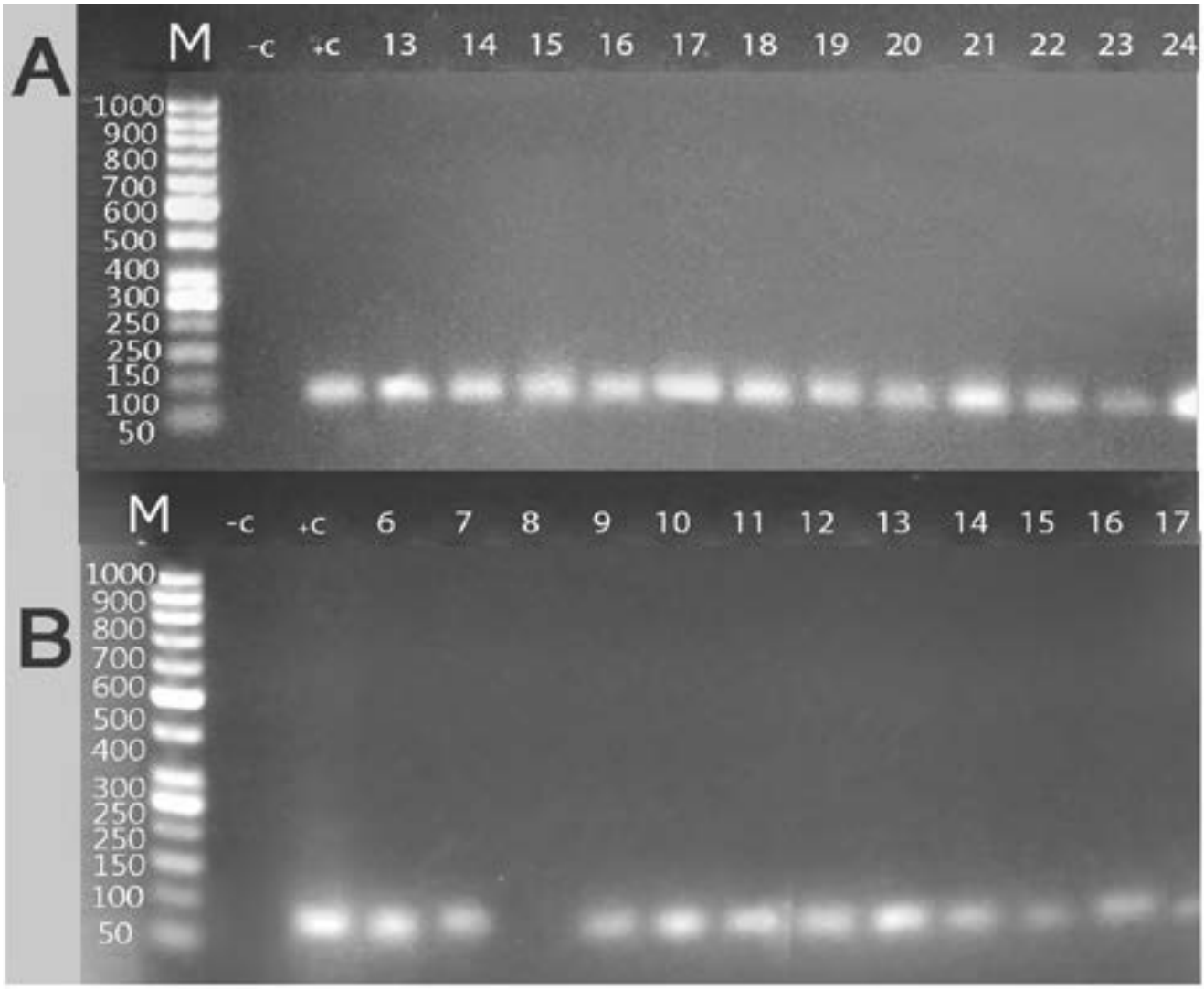

Fig. 2: Electrophoretic graph of conventional PCR products on 1.5% agarose gel stained with ethidium bromide in some represented *C. albicans* isolates (A: detection of Sap9 gene (80 bp amplicon), B: detection of Sap10 gene (80 bp amplicon). Lane 1 (M): represent 50 bp DNA ladder, Lane 2 (-C): negative control (reagent control mixture without DNA), Lane 3 (+C): positive control (reagent control mixture with DNA of standard strain ATCC 10231), Lane 4 to lane 15: clinical *C. albicans* isolates. DNA was extracted from Clinical *C. albicans* isolated from patients with systemic *Candida albicans* infections (respiratory tract, urinary tract infections and candidemia).

The data presented in table (2) show that 5 isolates out of 53 isolates were resistant to 3 anti-fungal which have proteolytic activity with prevalence of either Sap9 or Sap10 genes or both; out of these isolates 4 isolates show strong biofilm formation. The rest of isolates (48 isolates) which were resistant to 2 or 1 anti-fungal drugs show strong biofilm formation in only 8 isolates (16%), while 30 isolates have proteolytic activity (65%).

Table 2

Isolate No.	Source	Biofilm activity	Sap activity	Sap 9 gene	Sap 10 gene	resistance profile
ST	ATCC 10231	W	+	+	+	5-FC
1	R	N	+	+	+	5-FC
2	R	N	-	+	+	5-FC
3	R	N	++	+	+	5-FC
4	R	S	++	+	+	5-FC, FLU, VOR
5	R	N	+	-	+	5-FC
6	R	N	-	-	+	5-FC
7	R	N	++	+	+	5-FC, FLU, VOR
8	R	N	+	+	-	5-FC
9	R	N	-	+	+	5-FC, VOR
10	R	N	+	+	+	5-FC
11	B	N	++	+	+	5-FC
12	R	N	-	+	+	5-FC
13	R	N	-	+	+	5-FC
14	R	N	-	+	+	5-FC
15	R	N	+	+	+	5-FC
16	R	W	-	+	+	5-FC
17	R	N	-	+	+	5-FC
18	R	W	+	+	+	5-FC
19	R	N	+	+	+	5-FC
20	R	W	-	+	+	5-FC
21	B	N	+	+	+	5-FC
22	R	N	-	+	+	5-FC
23	R	N	-	+	+	5-FC
24	B	N	+	+	+	5-FC
25	R	N	-	+	+	5-FC
26	R	W	+	+	+	5-FC
27	R	N	+	+	+	5-FC
28	R	S	++	+	-	5-FC, FLU, VOR
29	R	W	-	+	-	5-FC
30	B	N	+	+	-	5-FC
31	R	N	+	+	-	5-FC
32	R	N	-	+	-	5-FC
33	R	N	+	+	+	5-FC
34	R	N	-	-	+	5-FC
35	B	N	-	+	+	5-FC
36	B	S	+	+	+	5-FC
37	U	W	+	+	+	5-FC
38	U	S	+	+	-	5-FC, FLU, VOR
39	U	S	+	+	+	5-FC
40	U	M	+	+	+	5-FC
41	U	S	+	+	+	5-FC
42	U	S	+	+	+	5-FC
43	U	W	-	-	-	5-FC
44	U	S	+	-	-	5-FC
45	U	S	+	-	+	5-FC
46	U	S	++	+	+	5-FC
47	U	N	++	+	+	5-FC
48	U	N	+	+	+	5-FC
49	U	W	+	-	+	5-FC
50	U	N	-	+	-	5-FC
51	U	W	+	-	-	5-FC
52	U	S	++	+	+	5-FC
53	U	S	+	+	+	5-FC, FLU, VOR

Discussion

The aim of this work was to determine the relationship between the anti-fungal susceptibility in one side, and the biofilm formation as well as the secretion of aspartic proteinases on the other side which represent major determinants associated with the pathogenicity of *Candida* species.

The available therapies against fungal diseases are limited and classified into five classes that have different targets in the fungal cells; polyenes, azoles and allylamines target cell membrane, while pyrimidine analogs target the fungal DNA and RNA. In contrast the new class echinocandins target the fungal cell wall[26].

Prolonged usage of azole anti-fungals in treating infections caused by *C. albicans* has led to the emergence of resistance. The acquisition of azole resistance in clinical isolates of *C. albicans* generally results in Multi-Drug (MDR) Resistance[27,28].

The presence of MDR in fungal pathogens is a serious complication during treatment of opportunistic fungal infections and poses a vital threat to the present therapeutic regimes by limiting the number of clinically useful anti-fungal drugs[29].

Biofilm formation in *C. albicans* has a major rule in pathogenesis which allows *Candida* to adhere to mucosal cells and polymeric surfaces of medical devices leading to spread of nosocomial infections. Biofilm forming cells are characterized by a three-dimensional structure that can survive the immune system of the host and associated with increasing the resistance to anti-fungal drugs[30]. The mechanism of biofilm resistance to anti-microbial agents is not fully understood. One hypothesis supposed that the formation of polysaccharide matrix has negative effect on the drugs penetration to fungal cells by formation of strong barrier[31], and only the outer layers are in contact with lethal doses of anti-fungals[5].

Interestingly, a correlation between anti-fungal susceptibility and biofilm formation was observed in four out of the five resistant clinical isolates in this work. These isolates were strong biofilm producers and revealed resistant to 3 anti-fungal drugs which in agreement with that reported by Bitar et al.[32], and Nobile and Mitchell[33]. They confirmed that the high resistance to fluconazole was usually associated with biofilm formation in *C. albicans* isolates. This correlation could be explained based on many mechanisms including active extrusion by efflux pumps[34].

Severe candidiasis is correlated to the production of extracellular hydrolytic enzymes which have a vital role in the pathogenesis of the yeasts[35,36].

Aspartyl proteases are one of the major virulence factors in *C. albicans* that reported to have a role in tissue invasion, hyphal formation, adherence, and phenotypic switching[10]. Many evidences prove the role of Sap enzymes in pathogenicity which include; the infected patients with *C. albicans* have higher proteolytic activity than asymptomatic carriers, in the other hand, the HIV infected patients with *C. albicans* have high proteolytic activity[10]. Severe candidiasis is correlated to the production of extracellular hydrolytic enzymes which have a vital role in the pathogenesis of the yeasts[35,36].

Furthermore a correlation between reduced susceptibility to anti-fungal agents and aspartyl proteinase production was confirmed in this study where all resistant isolates to the anti-fungals under investigations were aspartyl proteinase producers. In addition 80% of these isolates were biofilm producers.

The current study revealed other correlation between prevalence of Sap9 and Sap10 genes and the strong biofilm producers by *C. albicans* isolates, as 66.7% of these isolates have both Sap9 and Sap10, while 25% have either Sap9 or Sap10. One isolate (8.3%) has neither Sap9 nor Sap10. Previous result was consistent with that reported by Chaffin[37] and Schild et al.[17], in which the Sap9 and Sap10 mediate biofilm formation. Silva et al.[38], proved that pathogenicity is related to biofilm formation which is a likely indicator of growth, production of hydrolytic enzymes as well as resistance to anti-microbial activity. Furthermore, Rajendran et al.[39] found a correlation between higher SAP productions and increase the adhesion to buccal cells as well as resistance to fluconazole of *C. albicans* isolates.

Another study made a correlation between the extracellular proteolytic activity in vitro and the virulence of *Candida species* and prove that only the most virulent species such as *C. albicans*, *C. tropicalis* and *C. parapsilosis* produce more proteinases in vitro than do less virulent species[40].

Conclusion

Our study represents an advance in biomedical and health science by observing a link between the reduction in susceptibility to anti-fungal drugs and the virulence indicated by pathogenic *C. albicans* isolates mainly including the biofilm formation and Sap production.

Disclosure statement

No potential conflict of interest is reported by the authors.

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